The effect of Silver and Graphene Oxide nanoparticles on the growth behaviour of *Escherichia coli*

A PROJECT THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF TECHNOLOGY IN BIOTECHNOLOGY

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CERTIFICATE

This is to certify that project entitled "**The effect of Silver and Graphene Oxide nanoparticles on the growth behaviour of *Escherichia coli*"** submitted by SESAN NAYAK (Roll No. – 111BT0023), in partial fulfilment of the requirements for the award of Bachelor of Technology in Biotechnology at National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by him under my supervision and guidance. To the best of my knowledge, the matter embodied in the Project report has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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ABBREVIATIONS

LB	Luria-bertani
E.coli	*Escherichia coli*
AgNPs	Ag Nanoparticles
GO	Graphene oxide
ROS	Reactive oxygen species
OD	Optical Density
Conc	Concentration
Ag	Silver
gm	gram
mg	milligram
ml	milliliter
hr	Hour

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Abstract

The antibacterial study using nanoparticles (NPs) have many advantages such as the use of alternate of antibiotic-resistant bacteria. Although much information have been garnering, many more is still required. In this investigation, *Escherichia coli* was cultured in standard LB nutrient broth with two different NP species, Graphene oxide and Silver NPs under varying concentration. The growth of bacteria was expressed by measuring of the culture absorbance at 600 nm. The results demonstrated that silver NPs inhibited the bacterial growth, while Graphene oxide (GO) showed the enhancement of bacterial growth.

Key words: *Escherichia coli*, Silver nanoparticles, Graphene oxide nanoparticles, Spectrophotometer, Absorbance, Growth profile.

Chapter 1

Introduction

1.1 Nanoparticles

Matters at the range below 100nm start to show properties that are significantly different that of bulk matters. The change occurs in their physical, chemical and optical behaviour, which makes them quite different from their bulkier counterparts. The major reason is their high surface area to volume ratio. These change of properties are now being researched upon and exploited in many different spheres of science. These include biological sciences, electronics, automobile industry, chemical industry, in cosmetics, food industry etc. With number of fabrication and characterization techniques emerging for nanomaterials, many different types of nanomaterials are now in existence. These include nanoparticles, nano-fibers, carbon nanotubes, nano films etc which are finding widespread applications and potential use in upcoming technologies. Nanoparticles are getting huge attention in biological sciences, especially the way they can affect cellular physiology.

1.2 Prokaryotes

Prokaryotes are the simplest life form that can inhabit any kind of environment which may not sustain any complex life. They can found anywhere, starting from human skin to the very bottom of the ocean, from polar ice to the hot springs, where temperature soars up beyond 100^{o}C. These microbes show huge diversity in their cell size, shapes, and physiology with some common features like:-

i) Absence of membrane bound organelles

ii) Lack of true nucleus (membrane bound nucleus)

iii) Do not form haploid gametes and diploid zyGOtes

iv) Much smaller than eukaryotes

1.3 E. coli

Escherichia coli (named after Theodor Escherich) is a common gram negative, rod shaped bacteria, which often inhabits lower intestine of endotherms. It is one of the most well studied bacterium. Its regard as a model organism, makes it indispensable for number of research domains like genetics and molecular biology. Due to a short doubling time (20 min), it allows study of successive generations in a short time. It is very safe to work with, especially for new comers. It can be easily grown under laboratory conditions and the lineage can be easily maintained. Despite being normally non-pathogenic, its interaction with human body and its physiology is similar to many prokaryotic pathogens. So it is used for correlational study of pathogenic prokaryotes.

1.4 Silver Nanoparticles

The use of silver NPs dates back to 9^{th} century AD, when it was used in lustre decorations. In modern times Ag Nps are one of the most studied nanoparticles and most researched upon. The numerous use of Ag Nps include catalysis in chemical reactions and optical sensors. But the major attribute of Ag Nps, which has been studied is their ability to control and suppress bacterial growth. Ag Nps are different from Ag ions, which are positive ions, while Ag Nps being single crystals.

1.5 Graphene Oxide Nanoparticles

Graphene oxide (GO) is a derivative of graphite obtained by strong oxidization. It is actually the monomolecular sheets of graphite oxide. It results when bulk graphite oxide is dispersed in basic solution. This is analoGOus to graphene, which is the single layer form of graphite. Since its inception graphene oxide has found its use in water purifications, coating against corrosive materials, battery manufacture, along with generating a lot of onGOing research related to cutting edge technologies.

1.6 Ultraviolet–visible spectroscopy

This technique involves the absorption or reflectance of light wave in ultraviolet-visible region of spectrum. The O.D. (Optical density) measurement follows Bear-Lambert law which states that the absorbance of a solution varies directly with the density of the absorbing species and the free path length. UV-Vis spectrophotometer is also used as detectors in many biological processes, like HPLC (High pressure liquid chromatography), analytical chemistry, structural biology etc. Spectroscopic analysis is common for solutions, though solids and gases can also be analysed.

1.7 Objectives:

- To study the growth profile pattern of *E.coli.*
- To visualize the effect of Graphene oxide and Silver NPs on *E.coli* culture at varied concentration.

- To have a comparative analysis of the effects of the two nanoparticles.

Chapter 2

Literature Review

Silver for a long time has been known as an antimicrobial agent. Silver derived compound are used in many bacteria inhibiting applications. Silver derived compounds are also used in treating burns to prevent infection. Number of silver salts or their derivatives have commercial application as antimicrobial agents.

At normal pH, bacterial cell wall is negatively charged. This due to the excessive presence of carboxylic groups. Silver being positively charged, electrostatic attraction accounts for its adhesion to bacterial surface. Again high surface to volume ratio facilitates the cytotoxicity of the NPs. Another study says that silver ions, which can get dispersed from silver NPs, gets deposited in cell wall and granules to inhibit cell division, along with damaging cellular contents (Brown, T. and D. Smith. 1976; Richards, R. M. E., H. A. Odelola, and B. Anderson. 1984). Another theory after silver NP antibacterial effect is production of ROS like hydroxyl radical and super oxide anion (Corinne et al. 2000). Another study states that killing capacity of NPs can be size dependent (Nilda V. et al. 2009).

The antimicrobial or cytotoxic attribute of Graphene oxide has been disputed throughout many publications. Graphene oxides is monolayer sheets of graphite oxide having unique attributes and surface characteristics (Geim, A et al. 2007; Novoselov, K. 2004). Some research have suggested of cytotoxic effect of GO on both microbial and mammalian cells (Hu, W. et al. 2010). But there are studies which state that GO can promote cell proliferation and adhesion (Chen, H.

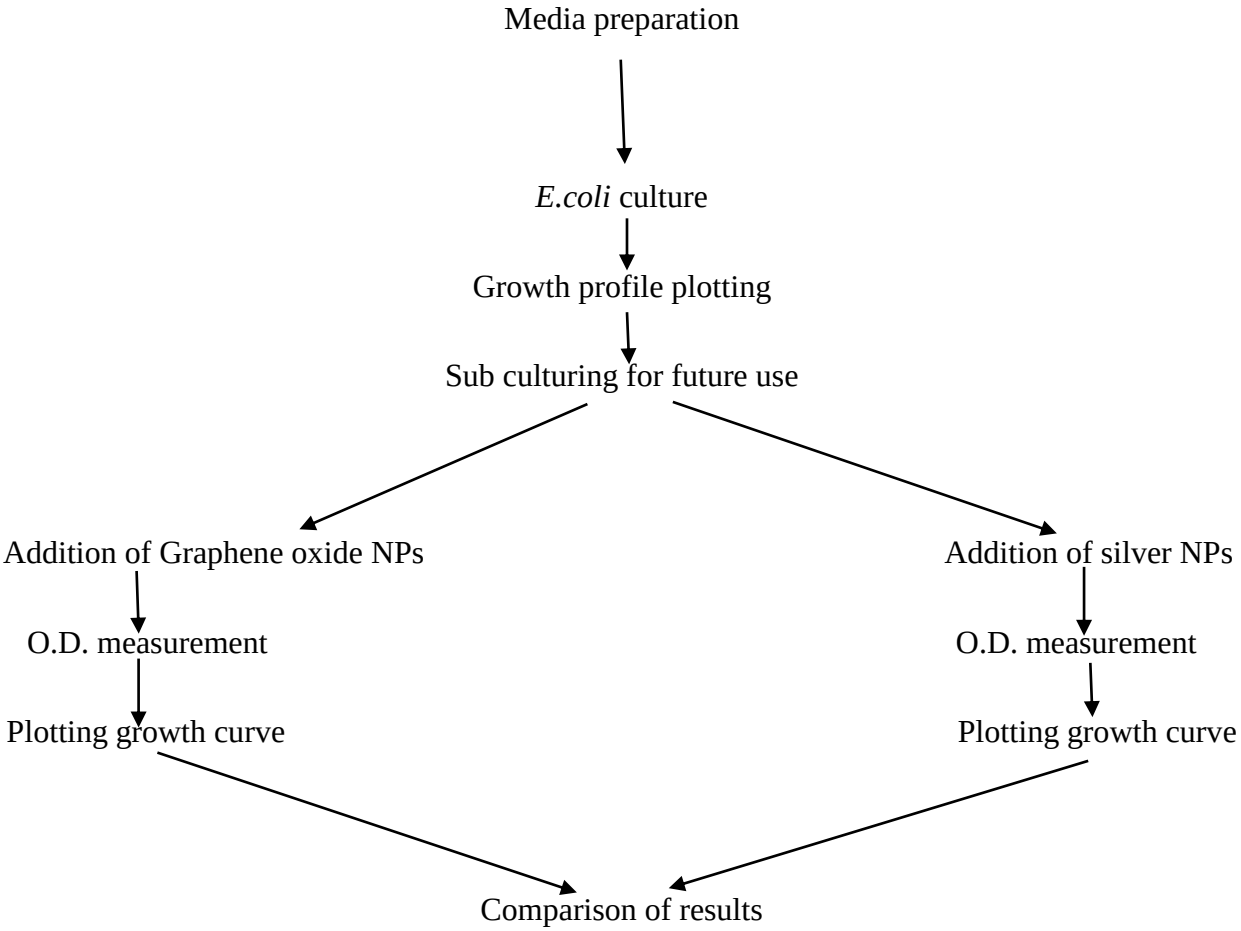
et al. 2010; Zhang, H. et al. 2010; Park, S. et al. 2010). Another study regarding GO's toxicity to microbes indicated that it is toxic to gram positive bacteria, but does not affect gram negative bacteria like *E.coli* (Kim et al. 2011). There are studies mentioning absence of any kind of antimicrobial effect of Graphene oxide (Das et al. 2011).There also has been reports stating strong cell growth enhancing property of GO in a nonspecific way (Ruiz et al. 2011).

In this study, DH5α strain of *E.coli* was used. It is a non-pathogenic strain, specially created for laboratory use. It was created by D. Hanahan as a cloning strain with multiple mutations enabling high efficiency of transmission (Taylor, R. G., Walker, D. C. and McInnes, R. R. ,1993). DH5α strain can be distinguished from other strains by examining the genetic sequence of its 16s small ribosomal subunits (Singh et al. 2010).

Chapter 3

Materials and Methods

3.1 Plan of work



3.2 Requirements:

Materials:

- *E. coli* strain (DH5α)
- Test tubes (10 ml, 20ml),Conical flasks (100 ml), Normal flasks
- LB media (From HIMEDIA)
- Deionized water

Instruments:

- Autoclave
- Laminar flow chamber
- Pipettes
- UV-Vis spectrophotometer
- Electronic balance
- Incubator

3.3 Media (Liquid broth) preparation

For *E.coli* culture LB media (Luria-Bertani) was used, which is a standard culture media. It constituents (gms/litre) are:-

Casein enzymatic hydrolysate	10.00
Yeast extract	5.00

Sodium chloride 5.00

Final pH of the media at 25^{o}C 7.0 $\pm$ 0.2

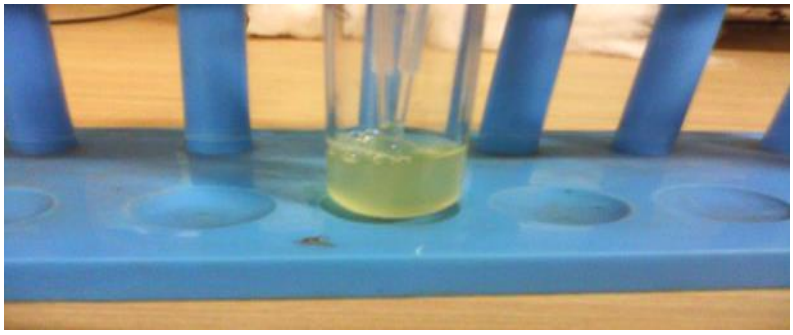
Protocol for making 50ml LB broth:-

- 1 gm of LB media was weighed in electronic balance
- 50 ml of deionized water was added to conical flask with 1gm LB media to make 50 ml broth
- The flask was cotton plugged and autoclaved at 121^{o}C for 20 mins
- The media was allowed to drop to normal temperature

After the preparation of the LB broth was prepared, it was inoculated with a sample of *E. coli* broth (gifted by Miss Usha Pandey) in the laminar flow chamber. Then the culture was incubated at 37^{o}C at 100 rpm.

LB Media after autoclaving (GOlden-yellow coloured)

The source *E.coli* culture



The turbid culture after significant bacteria growth

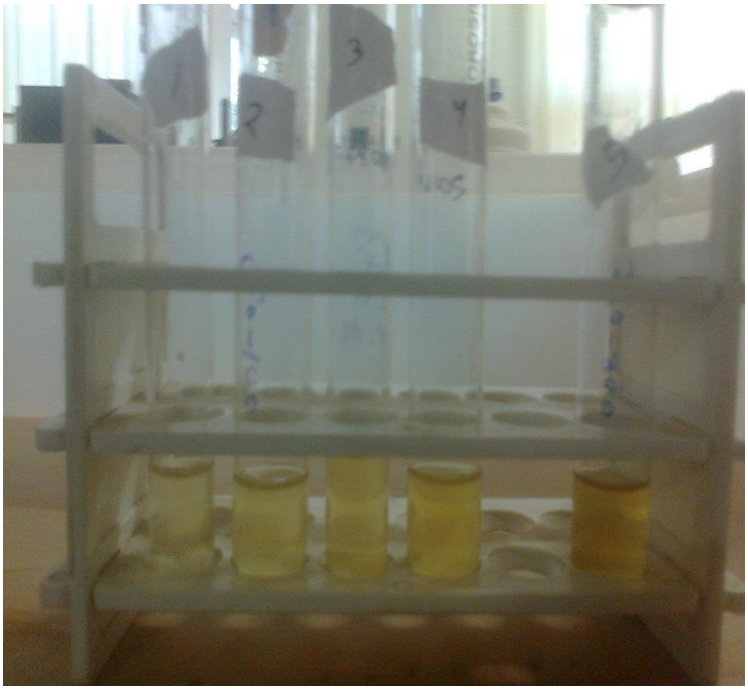
3.4 Nanoparticle Addition

Graphene Oxide

Graphene nanoparticles solution was gifted by Miss Usha Pandey (M.Tech, final year), which was produced by Pyrolysis method , with an initial concentration of 10mg/ml. The nanoparticles were of a range of 200 nm to 300 nm.

Graphene oxide NPs prepared by Pyrolysis method

GO addition to culture broth was performed in two sets of experiments. In both of the experiments, there were five cultures test tubes, numbered 1 to 5, containing 10 ml of media. To all the test tubes 200µl of inoculum was added. The no 1 test tube served as control, in which no NPs was added. In the 1st experiment the time intervals were roughly taken, just to observe the general effect of GO on *E. coli*. In 2nd experiment the time intervals and concentrations were synchronized with that of the corresponding silver NPs to have a comparative analysis.



The cultures after 36 hrs of growth

Silver Nanoparticles

Silver nanoparticles aqueous solution was gifted by Mr. Deependra K. Ban (Phd student). The original concentration was 10mg/ml and the solution prepared by citrate reduction method . The size of NPs were about 10nm.

Just like Graphene oxide, Silver NPs addition was performed 2 sets of experiments. For the 1st set of experiment, concentration of Ag NPs and time interval of O.D. measurement were roughly taken to know general effect of Ag NPs on *E.coli*. In the 2nd set of experiment all the parameters were synchronized with Graphene oxide experimentation for comparative analysis.

Test tubes having 10 ml LB media numbered 1 to 5 with their respective Ag NPs concentration

Chapter 4

Results

And Analysis

4.1 Growth profile measurement of *E. coli*

To make the growth profile of *E.coli*, standard 50 ml of standard LB broth was prepared. 1 ml of inoculum was added to it from a previous stock culture. The O.D. was taken every 2 hrs after the inoculation for 24 hrs at 600 nm. After 24 hrs, the growth profile was plotted from the collective O.D. readings. The readings are tabulated below.

Time in hours	**Optical Density**
2	0.0277
4	0.1245
6	0.1688
8	0.1748
10	0.1821
12	0.1943
14	0.1918
16	0.2006
18	0.2097
20	0.2128
22	0.2429
24	0.2417

The growth profiling was done by taking O.D. on X-axis and time (in hrs) on Y-axis.

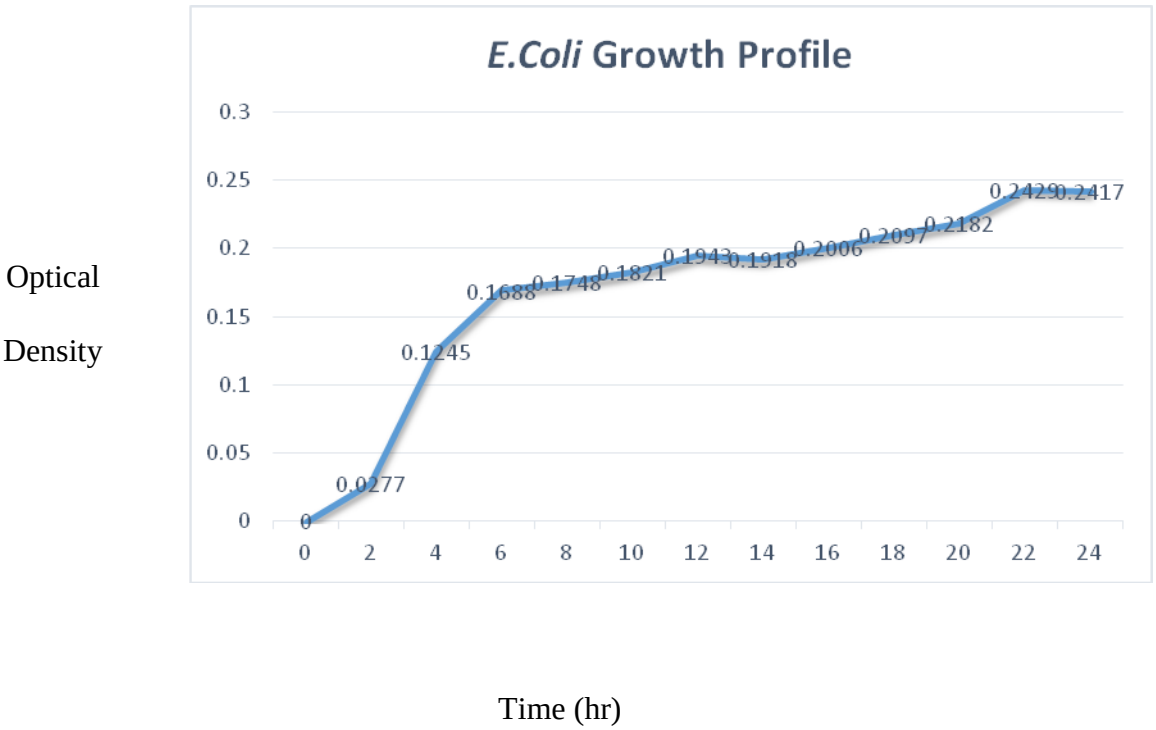


Figure 3.1: Growth Curve for *Escherichia coli* for 24 h.

From the growth curve, it was noticed that *E.coli* entered log phase around 3hr from inoculation, which persisted till 20 hr of inoculation. The duration and onset of log phase may vary with initial amount of inoculation.

4.2 Effect of Graphene Oxide on *E.coli*

In the 1st set of reading of GO NPs on *E.coli*, following GO concentrations were taken in the LB broth containing test tubes:

Test tube 1: 0 (Control)

Test tube 2: 0.05mg/ml

Test tube 3: 0.1mg/ml

Test tube 4: 0.2mg/ml

Test tube 5: 0.3mg/ml

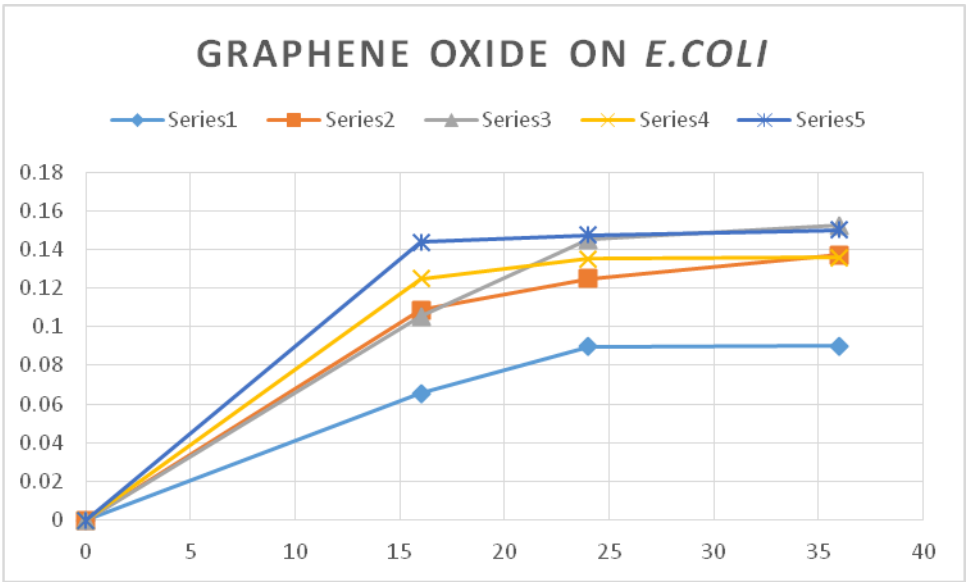
This experimentation was just done to visualize the general effect of GO on *E.coli*, with O.D. being taken at random time intervals. The results are tabulated below:

Table 2. Effect of GO on *E.coli* (at random time interval)

Time in hrs	OD (1)	OD (2)	OD (3)	OD (4)	OD (5)
16	0.0657	0.1086	0.1053	0.1247	0.1436
24	0.0897	0.1250	0.1451	0.1352	0.1474
36	0.0901	0.1371	0.1525	0.1357	0.1501

The following graph was plotted from the O.D readings

Graph 2: <u>Effect of GO on *E.coli* (at random time interval)</u>



O.D

Time (hrs)

During the O.D readings, the samples were diluted with blank. As it can be seen GO acts as an enhancer of cellular growth as the control is at the bottom. There is no steep rise in the plot as the O.D. were taken from 16 hrs, which might fall in the stationary phase.

4.3 Effect of Silver nanoparticles on *E.coli*

Just like GO, the 1st set of readings of Ag NPs on *E.coli* was done with random time intervals, just to find the general effect of Ag NPs on *E.coli*. The concentrations of the test tube were:

Test tube 1: 0 (Control)

Test tube 2: 0.065 mg/ml

Test tube 3: 0.13 mg/ml

Test tube 4: 0.26 mg/ml

Test tube 5: 0.39 mg/ml

The O.D. results are tabulated below:

Table 3. Effect of Ag NPs on E.coli at random time intervals

Time in hrs	OD (1)	OD (2)	OD (3)	OD (4)	OD (5)
6	0.1533	0.0959	0..0948	0.0911	0.0811
24	0.2177	0.1566	0.1416	0.1393	0.1308
30	0.2644	0.1424	0.1319	0.1322	0.1264

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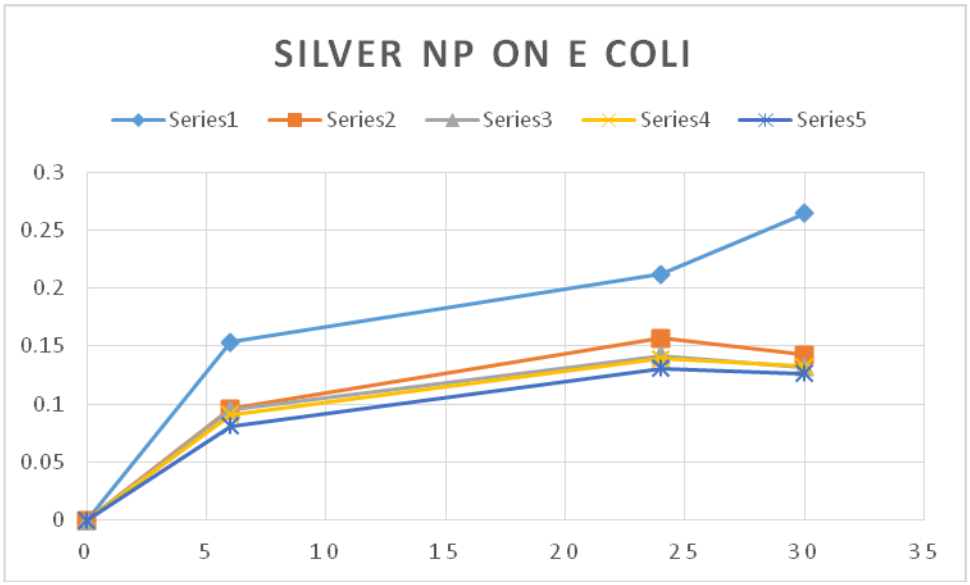
Graph 3: <u>Effect of Ag NPs on E.coli (at random time interval)</u>



O.D

Time (hrs)

From the plot, it is evident that Ag NPs inhibit bacterial growth. In this experimentation it cannot be said that silver nanoparticles has strong bactericidal activity, but it does slow down bacterial growth as the control is at the top. Here there is no steep decrease in growth with increase in Ag NPs concentration increase, but there is apparent decrease in growth with increasing concentrations.

4.4 Comparative analysis of Graphene oxide and Silver nanoparticles

In this experimentation, all the GO and silver concentrations variations were same, with O.D. taken at the same time intervals for both nanoparticles. The temperature and rpm during the incubation also were same. In both experimentation, the sample was diluted with blank prior to O.D. measurement.

For Graphene oxide:

Concentrations taken:

Test tube 1: control

Test tube 2: 0.05mg/ml

Test tube 3: 0.1mg /ml

Test tube 4: 0.2mg/ml

Test tube 5:0.3mg/ml

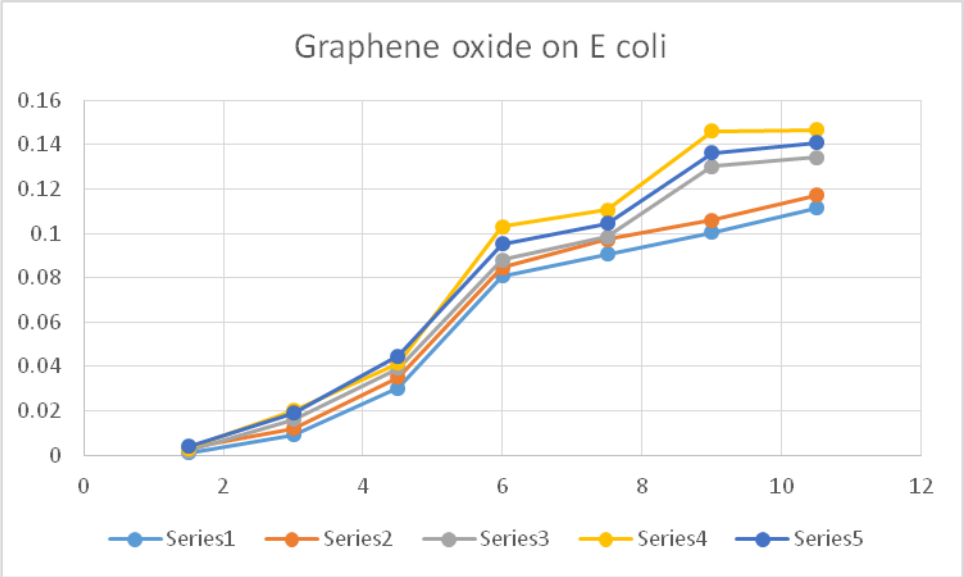
The O.D. were taken at an interval of 1.5 hrs after inoculation seven times to plot the profile. The results are tabulated below:

Table 4. Graphene oxide on E.coli (regular time intervals)

Time in hrs	OD (1)	OD (2)	OD (3)	OD (4)	OD (5)
1.5	0.001	0.003	0.002	0.003	0.004
3.0	0.009	0.012	0..016	0.020	0.019
4.5	0.0303	0.0352	0..0391	0..0415	0.0447
6.0	0.0807	0.0846	0.0881	0.1030	0.0953
7.5	0.0907	0.0972	0.0983	0.1107	0.1046
9.0	0.1005	0.1059	0.1302	0.1462	0.1363
10.5	0.1115	0.1172	0.1342	0.1467	0.1409

Graph 4: <u>Effect of GO on E.coli (at regular time interval)</u>

O.D.



Time (hrs)

Here it is obvious that Graphene oxide possess cell supportive nature. E.coli growth is enhanced with the increasing concentration of GO, though here 0.2 mg/ml gives more growth than 0.3 mg/ml, but there is no growth retardation.

For Silver nanoparticles:

Same concentrations of NPs were taken in the same fashion, with the cultures maintained at the same temperature and rpm as that of Graphene oxide. The concentrations were as follows:

Test tube 1: control

Test tube 2: 0.05mg/ml

Test tube 3: 0.1mg /ml

Test tube 4: 0.2mg/ml

Test tube 5:0.3mg/ml

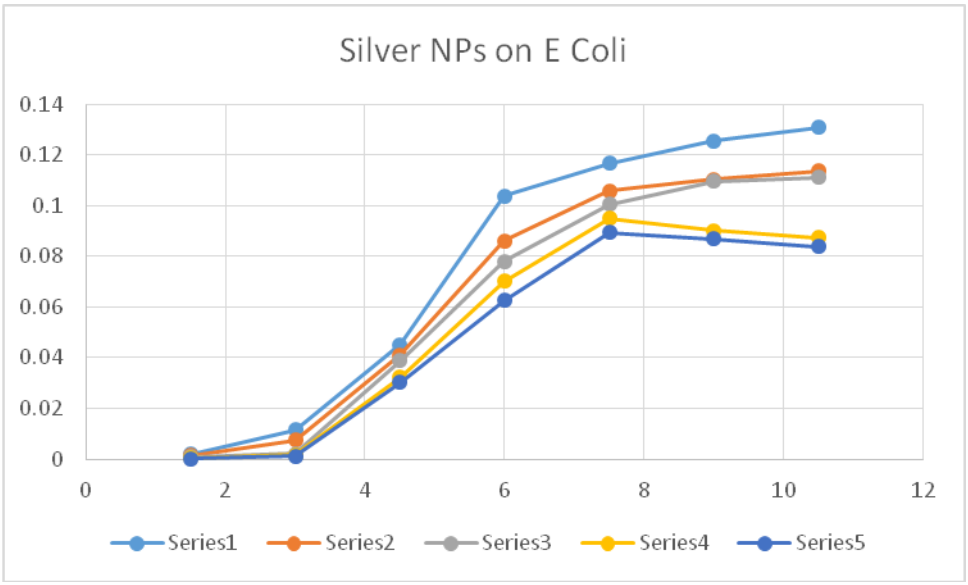
The O.D. was taken at 1.5 hrs of interval just like before and the results were tabulated.

Table 5. Silver nanoparticles on E.coli (regular time intervals)

Time in hrs	OD (A)	OD (B)	OD (C)	OD (D)	OD (E)
1.5	0.0019	0.0014	0.0010	0.0005	0.0002
3.0	0.0114	0.0075	0.0022	0.0016	0.0012
4.5	0.0452	0.0411	0.0388	0.0322	0.0302
6.0	0.1038	0.0862	0.0780	0.0703	0.0626
7.5	0.1167	0.1059	0.1006	0.0950	0.0895
9.0	0.1255	0.1105	0.1097	0.0901	0.0870
10.5	0.1310	0.1137	0.1112	0.0875	0.0838

Graph 5: <u>Effect of Silver nanoparticle on E.coli (at regular time interval)</u>

O.D.



Time (hrs)

From the profile it is evident that Ag NPs hinder bacterial growth. In this experimentation it has been seen that initially Ag NPs added cultures show stunted growth relative to the control, but still continue to grow. With time their growth rate decreases, while at higher concentrations (0.2mg/ml, 0.3mg/ml) growth shows a negative profile.

Chapter 5

Conclusion

5.0 CONCLUSION:

This study showed the effect of two different types of nanoparticles on *E.coli* at increasing concentration. The results concluded that Graphene oxide facilitated bacterial growth, while silver NPs inhibited the growth. The enhancing effect of GO varied directly with the concentration, though it is presumed that there might be a limit at which increase of GO concentration may not support bacterial growth anymore and may get detrimental. It is also noticed that its enhancing effect might be growth phase specific, that means it just facilitates bacterial growth and might not be the direct cause of it. With silver nanoparticles, it can be strongly said that the inhibition was concentration dependent. At small concentrations (0.05mg/ml, 0.1mg/ml), Ag NPs showed bacteriostatic properties and at higher concentrations (0.2 mg/ml, 0.3mg/ml) negative growth was obtained.

5.1 Future works:

This was a study which involved monitoring the effect of two different types of nanoparticles (GO and Ag NPs) on *E.coli* growth. Based on our current results we can find the following future work:

- Determination of cell supportive nature of GO for human cells and in case of positive results subsequent GO coated or composite scaffold fabrication.
- Determination of antibacterial effect of Ag NPs composites with other materials (other NPs or polymer) for controlled release and better effect on bacterial inhibition
- Similar experiment and comparative studies on eukaryotic microbes.

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